

Research Paper

## Identification of genes expressed by *Cryptococcus gattii* during iron deprivation

Daphine Ariadne Jesus de Paula<sup>1</sup>, Lívia Kmetzsch Rosa e Silva<sup>2</sup>,  
Charley Christian Staats<sup>2</sup>, Marilene H. Vainstein<sup>2</sup>, Ana Lúcia Pinto Joanoni<sup>1</sup>,  
Luciano Nakazato<sup>1</sup>, Valéria Dutra<sup>1</sup>

<sup>1</sup>Universidade Federal de Mato Grosso, Cuiabá, MT, Brazil.

<sup>2</sup>Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

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### Abstract

*Cryptococcus neoformans* and *C. gattii* are pathogenic yeasts that cause life-threatening diseases in humans and animals. Iron is an essential nutrient for virtually every organism as it functions as a cofactor in numerous essential enzymatic reactions. In the literature, the competition for iron between microbes and mammalian hosts during infection is well documented. In this study, we used representational difference analysis (RDA) in order to gain a better understanding of how *C. gattii* responds to iron starvation. A total of 15 and 29 genes were identified as having altered expression levels due to iron depletion after 3 h and 12 h, respectively. Of these, eight genes were identified in both libraries. The transcripts were related to many biological processes, such as cell cycle, ergosterol metabolism, cell wall organization, transportation, translation, cell respiration and the stress response. These data suggest a remodeling of *C. gattii* metabolism during conditions of iron deprivation.

**Key words:** *Cryptococcus gattii*, iron, representational difference analysis, yeast.

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### Introduction

The *Cryptococcus neoformans* species complex comprises basidiomycetous yeasts that cause life-threatening diseases in humans and animals. There are two main pathogenic species within the *Cryptococcus* genus, namely *C. neoformans* and *C. gattii*. *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* have been isolated worldwide, and typically cause disease in hosts with impaired immunity. *Cryptococcus gattii* is found in tropical and subtropical climates and is frequently associated with the infection of immunocompetent hosts (Kwong-Chung and Bennett, 1992; Sorrell 2001). *C. gattii* emerged as an important pathogen when an outbreak of cryptococcosis occurred in 1999 on Vancouver Island, British Columbia, Canada, and is still ongoing (Byrnes *et al.*, 2010; Kidd *et al.*, 2004; Stephen *et al.*, 2002), along with an outbreak of infections in otherwise healthy people and animals in western North America (Bartlett *et al.*, 2008). *C. gattii* therefore continues to pose serious public health problems for immunocompromised and healthy individuals worldwide.

The documented virulence factors are common to both *C. neoformans* and *C. gattii*, and include production of a polysaccharide capsule; formation of the pigment melanin in the cell wall; growth at 37 °C; and secretion of enzymes such as phospholipase B and urease (Casadevall and Perfect, 1998).

Iron is an essential nutrient for virtually every organism because it participates as a cofactor in numerous essential enzymatic reactions involving the transfer of electrons. In the literature, the competition for iron between microbes and mammalian hosts during infection is well documented (Sutak *et al.*, 2008). In mammalian hosts, the majority of iron is locked within iron-binding proteins, pathogens must therefore be equipped with competitive iron acquisition and uptake systems. *C. neoformans* possesses cell surface reductases that reduce ferric iron to its ferrous state (Cfo1), export reductants, such as 3-hydroxyanthranilic acid, and iron permease (Cft1) for transport into the cytosol as ferric ion (Jacobson *et al.*, 1998; Jung *et al.*, 2008b, 2009; Nyhus *et al.*, 1997, 1999). In addition, for *C. neoformans*, many studies have described genes related to iron regulation, in-

cluding *Cryptococcus* iron regulator 1 (CIR1) (Jung *et al.*, 2006) and HAPX, genes that control the expression of iron-dependent genes (Jung *et al.*, 2010).

In *C. gattii*, little is known about iron metabolism and its implication in virulence. Crestani *et al.* (2012) recently carried out a study of the proteomic profile of this yeast under iron-deplete and iron-replete conditions, and observed a similar regulation of metabolic proteins in both the pathogenic yeasts, *C. gattii* and *C. neoformans*. However, certain proteins related to iron homeostasis in *C. neoformans* were not identified. Silva *et al.* (2011) performed a genome comparative analysis of genes related to micronutrient metabolism in *C. neoformans* and *C. gattii*; these authors reported differences between the yeasts in expression of genes related to iron homeostasis, such as metalloredutase and ferroxidase homologs. Ma *et al.* (2010) proposed that the hypervirulence exhibited by *C. gattii* is associated with its mitochondrial gene expression.

Herein, we were interested in gaining an understanding of the mechanisms involved in iron regulation in *C. gattii*. In order to investigate the adaptive cellular responses when host-iron availability to pathogenic microorganisms is reduced, we attempted to identify the differential gene expression profile of the *C. gattii* reference strain R265 under conditions of iron deprivation, using representational difference analysis (RDA).

## Materials and Methods

### Strain, culture conditions and RNA extraction

The *C. gattii* strain R265 was used for the RDA experiments and gene expression analysis. For construction of the RDA libraries, R265 was routinely grown in Yeast Peptone Dextrose (YPD) broth (yeast extract 1%, peptone 1% and glucose 2%) prior to cultivation in medium containing low levels of iron (limited-iron medium (LIM), according to Jacobson *et al.* (1998) and an iron-repleted medium (LIM+Fe, with the addition of 100 $\mu$ M FeHEDTA, Sigma Chemical Co., St Louis, MO) for 24 h. To evaluate the effects of iron, 10<sup>6</sup> cells/mL of yeast were transferred to 50 mL of LIM or LIM-Fe. Cells were grown for two distinct periods of time (3 h or 12 h) in LIM or LIM-Fe at 37 °C. Salts of polyvalent metals were dissolved in water treated with Chelex-100 (Bio-Rad) and filter sterilized. All glassware was soaked in Citranox acid detergent overnight and rinsed with distilled, deionized water before use with LIM.

Cells were harvested by centrifugation and immediately frozen in liquid nitrogen prior to RNA extraction. Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany), and cDNA was synthesized using the SMART PCR synthesis kit (CLONTECH Laboratories, Palo Alto, CA). First-strand cDNA synthesis was performed with reverse transcriptase (RT M-MLV, Invitrogen, Carlsbad, CA) from 500 ng of total RNA. An aliquot

of 5  $\mu$ L of first-strand cDNA was used as the template for second-strand synthesis.

### Representational difference analysis

RDA was performed according to the protocol previously described by Dutra *et al.* (2004). Subtracted libraries were constructed using cDNA from *C. gattii* grown for 3 h and 12 h in a low-level iron medium (LIM) as the “tester” and cDNA from *C. gattii* grown for 3 h and 12 h in iron-replete medium (LIM+Fe) as the “driver”. All cultures were grown at 37 °C with shaking. A double-stranded cDNA sample from each experimental condition was digested with *Sau3AI* (Sigma-Aldrich, St Louis, MO, USA) and resulting products were purified using the illustra GFX PCR DNA and gel band purification kit (GE Healthcare, Chalfont St Giles, England). The RBam24/12 adapters were ligated to the digested cDNA in order to be used as a tester. The first differential product (Dp1) was obtained by hybridization (20 h at 67 °C) of the driver and cDNA mixed at a 10:1 ratio, followed by PCR amplification with an RBam24 primer. In order to generate the second (Dp2) and third (Dp3) differential products, NBam and JBam adapters were ligated to the tester in each round of subtractive hybridization and the driver/tester ratio was increased to 100:1 and 1000:1, respectively. The oligonucleotides used for the RDA are listed in Table S1.

### Cloning and bioinformatics analysis of the RDA products

The final RDA products (Dp3) were blunted with a Klenow DNA polymerase I fragment (Invitrogen) and phosphorylated with T4 polynucleotide kinase (Invitrogen). Fragments were ligated to a *SmaI*-digested and dephosphorylated pUC18 vector. *Escherichia coli* strain XL1-Blue-competent cells were transformed with the ligation products, and plasmid DNA was prepared from selected clones. Cloned inserts were sequenced using the DYEnamic ET Dye Terminator Cycle sequencing kit for the MegaBACE DNA analysis system (GE). Sequence quality was analyzed and the contig assembly was constructed using the Staden Package Software. Resulting sequences were compared to the Broad Institute Database (<http://www.broadinstitute.org>). Sequences returning matches with an E-value  $\leq -10$  were annotated and classified based on their putative molecular function and/or biological process using the Gene Ontology classification system. Searches for well-conserved functional motifs were performed using the NCBI - conserved domain database (CDD).

### Quantitative real-time RT-PCR

Primers for real-time RT-qPCR analysis were designed using Oligo Explorer 1.1.0 and are listed in Table S1. The predicted product length varied between 150 bp and 200 bp. Real-time RT-qPCR was performed us-

ing a StepOne™ Real-Time PCR System (Applied Biosystems). Reactions were composed of Platinum SYBR Green qPCR Supermix (Invitrogen), using 5 pmol of each primer, 2 µL of template at a final volume of 25 µL. The PCR thermal cycling conditions were as follows: an initial step at 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 30 s. Melting curve analyses were performed at the end of the reaction to confirm single PCR products. PCR reactions were monitored, and relative gene expression was quantified based on the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001). To attest the yeast iron-depleted conditions, the Hap proteins (HapX), transcription factor (Cir), iron permease (Cft1) and ferroxidase (Cfo1) targets were used in RT-qPCR experiments (Table S1). The housekeeping gene, actin (act), was used for normalization. All experiments were carried out using two independent cultures, and each cDNA sample was analyzed in triplicate for each target gene, as well as in triplicate for the housekeeping gene.

## Results

A total of 389 and 442 high quality sequenced cDNA clones were obtained after 3 h and 12 h RDA, respectively. Through the RDA methodology, we found a total of 36 genes with differential RNA levels, where 15 genes were differentially expressed at 3 h, and 29 genes were differentially expressed at 12 h; of these, eight genes were identified in both libraries. Functional profiling of these genes using the Gene Ontology classification revealed transcripts10 and 13 as being the main groups for the libraries (Tables 1 and 2). These transcripts were related to many different biological processes. We confirmed, by the RT-qPCR technique, that the upregulated transcripts encoded for c-4 methyl sterol oxidase, alpha tubulin, polyadenylate-binding protein, transcriptional activator, vacuolar membrane protein, and Rds1 in the 3 h library (Table 1). Regarding the transcripts obtained from the 12 h library, c-4 methyl sterol oxidase, ubiquitin-protein ligase, transporters, diacylglycerol cholinephosphotransferase, heat shock protein 90, Rds1, polyadenylate-binding protein, glucosidase, alpha tubulin and nucleus protein were all upregulated (Table 2).

As we found some conserved hypothetical proteins in the present study, we performed searches in the NCBI Conserved Domains Database (CDD) in order to find the presence of domains that may provide insights into the function of these proteins. The hypothetical protein with the GenBank accession number XP\_570947.1 has a ZZ-type zinc finger domain, thought to be involved in protein-protein interactions. The hypothetical protein XP\_776919.1 has an *inositol monophosphatase* (IMPase) domain and other related domains. IMPase dephosphorylates inositol phosphate to generate inositol, which may

be recycled into inositol lipids; in eukaryotes, IMPase plays a vital role in intracellular signaling.

We also found a *dual specificity phosphatase* catalytic domain (Ser/Thr and Tyr protein phosphatase) at the hypothetical XP\_570177.1. These are tyrosine-specific protein phosphatases that catalyze the removal of a phosphate group attached to a tyrosine residue using a cysteinyl-phosphate enzyme intermediate. These enzymes are key regulatory components in signal transduction pathways (such as the MAP kinase pathway), cell cycle control and the control of cell growth, proliferation, differentiation and transformation.

## Discussion

We describe an application of the RDA methodology in the search for genes potentially relevant in the pathogenesis of the human pathogenic microorganism *C. gattii*. This technique was applied to *C. neoformans* and *C. gattii* to detect new genes, possibly involved in disease pathogenesis (Goulart *et al.*, 2001; Rosa e Silva *et al.*, 2008).

The cellular machinery involved in iron metabolism has been more clearly demonstrated for *C. neoformans* (Jacobson *et al.*, 1998; Jung *et al.*, 2006, 2008a, 2008b, 2009, 2010; Jung and Kronstad, 2011; Lian *et al.*, 2005) compared to *C. gattii*, where only a proteomic profile by Crestani *et al.* (2012) and an in silico analysis performed by Silva *et al.* (2011) have been described.

*Cryptococcus neoformans* uses several transport systems to acquire iron from the environment. Both high- and low-affinity iron uptake activities, mediated by cell surface reductases, have been described, namely the permease/ferroxidase complex for iron uptake, iron sensing/regulation proteins and siderophore utilization (Jacobson *et al.*, 1998). Our RDA study revealed some different results in gene function compared with previous studies in *C. neoformans*. However, the studies mentioned above have been based on different approaches for evaluating gene expression; RDA methodology uses an alternative strategy and is a sensitivity tool for the isolation of rare differentially expressed transcripts (Pastorian *et al.*, 2000). In this way, it was possible to identify transcripts representing only 1% of the mRNA population of a cell (Sugita and Sugiura, 1994). Due the relatively abundant expression of genes such as reductases and the permease/ferroxidase complex under iron deprivation conditions, it is possible that the technique was not able to identify these genes.

We found an alteration in the transcript 5-amino-levulinate synthase; this gene was differentially expressed in the 3 h library. However, despite being identified in the 12 h library using the RDA technique, it was not subsequently confirmed by RT-qPCR. The enzyme 5-amino-levulinate synthase catalyzes the first step in the heme biosynthetic pathway, with many transcripts encoding components of respiratory cytochromes, which contain

**Table 1** - Summary of the computational analysis <sup>a</sup> of the transcripts obtained from *C. gattii* grown in a low iron medium for 3 h at 37 °C

| Biological process                       | E value <sup>b</sup> | Access number (Genbank) <sup>c</sup> | Frequency (number of clones) <sup>d</sup> | Fold-change |
|--|----------------------|--------------------------------------|---|-------------|
| Ergosterol metabolism                    |                      |                                      |   |             |
| C-4 methyl sterol oxidase                | 1E-115               | XP_569528.1                          | 93  | 1.30        |
| Cell cycle                               |                      |                                      |   |             |
| alpha tubulin                            | 2E-70                | XP_568869.1                          | 162                                       | 1.60        |
| g1/s-specific cyclin pcl1 (cyclin hcs26) | 9E-22                | XP_571897.1                          | 11  | 0.91        |
| MMS2                                     | 9E-16                | XP_567564.1                          | 04  | 0.81        |
| Translation                              |                      |                                      |   |             |
| polyadenylate-binding protein            | 3E-34                | XP_572834.1                          | 73  | 1.18        |
| Cell wall organization                   |                      |                                      |   |             |
| endoglucanase E-4 precursor              | 6E-73                | XP_572272.1                          | 17  | 0.88        |
| Secretion and vesicle trafficking        |                      |                                      |   |             |
| vacuolar membrane protein                | 5E-04                | XP_569704.1                          | 06  | 1.7         |
| ARF small monomeric GTPase               | 1E-10                | XP_572032.1                          | 01  | 0.81        |
| Cellular respiration                     |                      |                                      |   |             |
| 5-aminolevulinate synthase               | 9E-70                | XP_569249.1                          | 04  | 1.1         |
| Stress response                          |                      |                                      |   |             |
| Rds1 protein                             | 8E-11                | XP_568514.1                          | 01  | 2.0         |
| Transcription                            |                      |                                      |   |             |
| transcriptional activator                | 1E-34                | XP_566547.1                          | 01  | 1.3         |
| Others                                   |                      |                                      |   |             |
| Metallochaperone                         | 9E-09                | XP_572504.1                          | 05  | 0.86        |
| minor histocompatibility antigen h13     | 9E-32                | XP_572261.1                          | 09  | 0.70        |
| Hypothetical proteins                    |                      |                                      |   |             |
| hypothetical protein CNF04510            | 1E-18                | XP_571639.1                          | 01  | 1.35        |
| hypothetical protein CNJ02890            | 8E-18                | XP_567569.1                          | 01  | 1.52        |
| Control Iron genes <sup>e</sup>          |                      |                                      |   |             |
| high-affinity iron permease              |                      | AFR98470.1                           |   | 15.41       |
| Multicopper oxidase                      |                      | AFR98469.1                           |   | 17.45       |
| Ion regulator 1                          |                      | AFR97352.1                           |   | 1.19        |
| bZIP transcription factor                |                      | AFR94904.1                           |   | 8.21        |

<sup>a</sup>Sequence quality was analyzed and contig assembly was constructed by using the Staden Package Software. Resulting sequences were compared to the Genbank database using the BLASTX program.

<sup>b</sup>E value according to the information from BLASTX searches of the non-redundant database at NCBI.

<sup>c</sup>Accession number of the gene products in the GenBank database.

<sup>d</sup>Frequency represents the number of clones found in a total of 389 high quality sequenced cDNA clones.

<sup>e</sup>Genes used to validate iron deprivation conditions represented by reductive, high affinity iron transport and transcriptional factors).

several heme cofactors and some heme biosynthetic genes, and are down-regulated during iron deficiency (Shakoury-Elizeh *et al.*, 2004, 2010). Transcriptional profiles of genes encoding components of respiratory cytochromes, are also influenced by the availability of iron (Pouliot *et al.*, 2010). Under conditions of iron deficiency, the yeast activates the expression of proteins that lead to the degradation of mRNAs encoding proteins that function in iron-dependent pathways, thereby reducing the amount of iron required by these pathways (Sanguinetti *et al.*, 2006). Perhaps this explains the differential expression evident at 3 h but not upon

prolonged exposure to iron deprivation as observed in the 12 h library.

Genes controlling cell cycle, such as g1/s-specific cyclin pcl1 (cyclin hcs26), were found in this study. Cyclin expression can fluctuate according to the stage of infection and it is suppressed at distinct stages in the process (García-Muse *et al.*, 2004), and a similar scenario might take place in *C. gattii*. We observed that iron deficiency influenced the expression of these genes and, along with the reasons already mentioned, changes in the concentration of the ion might be another factor that could explain the reduction in the transcription of cell cycle-associated genes. In *C.*

**Table 2** - Summary of computational analysis <sup>a</sup> of the transcripts obtained from *C. gattii* grown in low iron medium for 12 h at 37 °C.

| Annotation                               | E value <sup>b</sup> | Access number (Genbank) <sup>c</sup> | Frequency (number of clones) <sup>d</sup> | Fold-change |
|--|----------------------|--------------------------------------|---|-------------|
| Ergosterol metabolism                    |                      |                                      |   |             |
| C-4 methyl sterol oxidase                | 2E-116               | XP_569528.1                          | 194                                       | 2.02        |
| Protein catabolism                       |                      |                                      |   |             |
| ubiquitin-protein ligase                 | 2E-37                | XP_572505.1                          | 11  | 1.40        |
| Cellular respiration                     |                      |                                      |   |             |
| mitochondrion protein                    | 7E-22                | XP_571658.1                          | 13  | 1.30        |
| 5-aminolevulinic synthase                | 7E-106               | XP_569249.1                          | 09  | 0.52        |
| Lipid metabolism                         |                      |                                      |   |             |
| Delta-12 fatty acid desaturase           | 2E-37                | XP_570226.1                          | 01  | 0.65        |
| Transporters                             |                      |                                      |   |             |
| ABC transporter                          | 5E-28                | XP_568169.1                          | 02  | 2.42        |
| inorganic phosphate transporter          | 1E-90                | XP_568544.1                          | 03  | 1.20        |
| mRNA export factor elf1                  | 9E-66                | XP_566522.1                          | 01  | 1.90        |
| Cell wall or membrane organization       |                      |                                      |   |             |
| diacylglycerol cholinephosphotransferase | 1E-71                | XP_571616.1                          | 21  | 2.60        |
| Stress response                          |                      |                                      |   |             |
| Heat shock protein 90                    | 7E-39                | XM_568451.1                          | 01  | 2.10        |
| Rds1 protein                             | 1E-05                | XP_568514.1                          | 08  | 5.70        |
| Translation                              |                      |                                      |   |             |
| polyadenylate-binding protein            | 4E-22                | XP_572834.1                          | 04  | 1.35        |
| ribosomal protein L1                     | 3E-48                | XP_569608.1                          | 01  | 0.85        |
| Carbohydrate metabolism                  |                      |                                      |   |             |
| Glucosidase                              | 2E-24                | XP_570389.1                          | 02  | 1.90        |
| Cell cycle                               |                      |                                      |   |             |
| g1/s-specific cyclin pcl1 (cyclin hcs26) | 7E-29                | XP_571897.1                          | 01  | 0.60        |
| alpha tubulin                            | 2E-57                | XP_568869.1                          | 02  | 1.20        |
| Nucleotide metabolism                    |                      |                                      |   |             |
| guanosine-diphosphatase                  | 2E-86                | XP_568971.1                          | 02  | 0.75        |
| Others                                   |                      |                                      |   |             |
| minor histocompatibility antigen h13     | 5E-34                | XP_572261.1                          | 01  | 0.80        |
| nucleus protein                          | 1E-28                | XP_567927.1                          | 11  | 1.0         |
| Hypothetical proteins                    |                      |                                      |   |             |
| hypothetical protein                     | 2E-24                | XP_571639.1                          | 7   | 0.71        |
| hypothetical protein CNBM2310            | 1E-60                | XP_772074.1                          | 80  | 2.44        |
| hypothetical protein CNBH2900            | 3E-42                | XP_773838.1                          | 02  | 0.87        |
| hypothetical protein CNBC2910            | 2E-23                | XP_776800.1                          | 01  | 0.30        |
| hypothetical protein                     | 2E-36                | XP_570947.1                          | 01  | -           |
| hypothetical protein CNBC4100            | 6E-23                | XP_776919.1                          | 01  | 1.85        |
| hypothetical protein                     | 4E-18                | XP_570177.1                          | 01  | 1.50        |
| hypothetical protein CNBM0150            | 4E-40                | XP_772247.1                          | 04  | -           |
| hypothetical protein CNM02470            | 5E-108               | XP_568428.1                          | 45  | -           |
| hypothetical protein CNBM0150            | 9E-33                | XP_772247.1                          | 12  | -           |
| Control Iron genes <sup>e</sup>          |                      |                                      |   |             |
| high-affinity iron permease CaFTR1       |                      | AFR98470.1                           |   | 22.15       |
| Multicopper oxidase                      |                      | AFR98469.1                           |   | 11.77       |
| Ion regulator 1                          |                      | AFR97352.1                           |   | 0.50        |
| bZIP transcription factor                |                      | AFR94904.1                           |   | 8.08        |

<sup>a</sup>Sequence quality was analyzed using the Phred Program, and contig assembly was performed by using the Staden Package Software. Resulting sequences were compared to the Genbank database using the BLASTX program.

<sup>b</sup>E value according to information from BLASTX searches of the non-redundant database at NCBI.

<sup>c</sup>Accession number of the gene products in the GenBank database.

<sup>d</sup>Frequency represents the number of clones found in a total of 442 high quality sequenced cDNA clones.

<sup>e</sup>Genes used to validate iron deprivation conditions represented by reductive, high affinity iron transport and transcriptional factors.

*gattii*, a pattern of alterations in expression of genes related to the cell cycle and the organization of cellular organelles has been described for the specifically strain R265 (Ngamskulrunroj *et al.*, 2011).

A slight regulation was demonstrated for an iron-regulated transcript identified in our study, which was shown to be a mitochondrial protein. It was also reported that iron availability affects mitochondrial-related genes in *C. neoformans* and proteins in *C. gattii* (Crestani *et al.*, 2012; Lian *et al.*, 2005). Ma *et al.* (2009) demonstrated a potential role of mitochondria in regulating virulence in the Vancouver Island Outbreak (VIO) lineage of *C. gattii*. The authors showed that *C. gattii* strains from within the VIO lineage exhibited enhanced intracellular proliferation within host macrophages and identified mitochondrial gene expression as the major hallmark of virulence in this species.

We found a novel transcriptional activator belonging to the GATA zinc finger gene family (XP\_566547.1) that was only upregulated in the 3 h library (1.3-fold). According to Jung *et al.* (2006, 2008a), Cir1, a GATA-type transcription factor, is not influenced by iron, although Cir1 mediates nearly all of the transcriptional responses to iron in *C. neoformans*. Cir1 regulates the expression of genes required for the utilization of essential metals such as iron and copper, the transcription of iron transport and homeostasis functions, and influences the main virulence factors (Jung *et al.*, 2006). *cir1* mutants displayed transcriptional unresponsiveness to iron deprivation, poor growth at 37 °C, loss of capsule formation, altered melanin production and were completely avirulent in a mouse model of cryptococcosis. Recently, Jung *et al.* (2010) described the role of HAPX genes in iron utilization and virulence in *C. neoformans*. Hap proteins, and specifically HapX, can regulate the expression of siderophore transporters and, in addition, can also positively regulate the expression of the CIR1 transcript. Here we find a novel transcriptional activator influenced by iron, but further studies must be carried out in order to determine how, as well as which genes this activator can regulate in response to iron deprivation.

In *C. neoformans*, ABC transporters are up-regulated in cells internalized by murine macrophages (Jung *et al.*, 2006), and ATP-binding cassette transporter-encoding gene antifungal resistance 1 (AFR1) enhances the virulence of *C. neoformans* and its ability to survive within macrophages (Sanguinetti *et al.*, 2006). In this study, the up-regulation of this transporter can be related to the stress response involved in the prolonged exposure time to iron deprivation conditions, as the gene only shows a different expression level in the 12 h condition. Perhaps, these iron deprivation conditions stimulate ABC transporters to mobilize stored iron. In *S. cerevisiae*, Atm1p is an ABC transporter located in the mitochondrial inner membrane that exports sulfur and possibly iron from the mitochondria to the cytosol (Chloupková *et al.*, 2004). Analysis in *S. pombe*

identified an *abc3+* (*ABCC* subfamily of ATP-binding cassette (ABC) transporters) (Paumi *et al.*, 2008) that was transcriptionally activated in response to iron deprivation. In response to iron deficiency (Pouliot *et al.*, 2010), *abc3+* could serve to transport iron stored from the vacuole to the cytoplasm.

The RDA data revealed a connection between iron deprivation, ergosterol metabolism, lipid metabolism and cell wall and membrane organization. *C-4 methyl sterol oxidase* was up-regulated at 3 h and 12 h in response to iron deprivation. Biosynthesis of sterol and of unsaturated fatty acids are essential processes that depend on both oxygen and iron. Philpott and Protchenko (2008) demonstrated that genes related to sterol biosynthesis (ERG genes) increased upon iron depletion. The authors suggest that the activity of iron-requiring proteins in these pathways is reduced, thereby limiting the levels of reaction products and resulting in decreased feedback inhibition and increased gene transcription. The connection between oxygen-sensing, ergosterol synthesis and iron uptake in *C. neoformans* may be due to the deletion of the regulatory gene *SRE1*, which downregulates genes required for ergosterol biosynthesis and for iron and copper transport (Chang *et al.*, 2007). The mutants *cir1* and *cf1* increased sensitivity to an inhibitor of ergosterol biosynthesis.

Diacylglycerol cholinephosphotransferase, a gene related to cell wall and membrane organization, was found to be induced under conditions of iron deprivation. In response to stress, cell remodeling via sterol metabolism and fatty acid biosynthesis has been described (Puig *et al.*, 2005). This gene is regulated by temperature in *C. neoformans* (Rosa e Silva *et al.*, 2008), and our results suggest that not only temperature, but also under iron deprivation conditions, remodeling of cell membranes may occur.

In summary, we found some novel genes related to iron homeostasis. However, further characterization of these genes through *in vivo* assays (gene knockout) is necessary in order to establish how these genes function, and how their regulation of iron levels influences *C. gattii* pathogenicity. Next Generation Sequencing (NGS) technologies are an alternative for qualitatively and quantitatively profiling the complete set of transcripts in order to fully understand the *C. gattii* response to iron and other micronutrient deficiencies.

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